'Affinity' Chromatography of Steroid-Transforming Enzymes with a Non-Steroidal Ligand

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The chromatographic behaviour of an avian oestradiol-17 β dehydrogenase, the 3(17) β -hydroxy steroid dehydrogenase from *Pseudomonas testosteroni* and cortisone reductase from *Streptomyces dehydrogenans* was studied on columns of *p*-(phenoxypropoxy)aniline attached to CNBr-activated Sepharose. The ligand was effective in adsorbing the oestradiol dehydrogenase from a partially purified extract of chicken liver, and the cortisone reductase was preferentially retained when mixtures of the three dehydrogenases were applied to columns in 10 mM-buffer. Under these conditions the 3(17) β -hydroxy steroid dehydrogenase was eluted in the front, but was adsorbed in the presence of 3 M-KCl. β -N-Acetylglucosaminidase present in the liver preparation was not retained by the ligand, whereas lactate dehydrogenase from rabbit muscle was adsorbed in a manner similar to the retention pattern found on affinity chromatography with 2',5'-ADP-Sepharose. The mean overall purification of the oestradiol dehydrogenase was 13-fold, with a mean recovery of 53%. *p*-(Phenoxypropoxy)aniline offers promise for the purification of steroid-transforming enzymes where elution with substrate or cofactor is not wanted. It is also suggested that the ligand may be of service in the purification of receptors for hormonal steroids.

Affinity chromatography is a technique of proven value and widespread application which has been exploited with varying degrees of success in steroid biochemistry for the isolation of steroid-binding plasma proteins, hormonal receptors and steroid-transforming enzymes. Thus Burstein (1969) removed testosterone-binding globulin in low yield from human plasma by means of an androstane derivative covalently bound to Sepharose 4B, and Rosner & Smith (1975) characterized testosterone-oestradiol-binding globulin from the same source, by attaching 5 α -androstane-3 β ,17 β -diol 3-hemisuccinate to Sepharose through an azodianiline leash.

Receptor molecules have been similarly purified, e.g. by Sica *et al.* (1973), Kuhn *et al.* (1975) and Coffey *et al.* (1977) using steroids linked to agarose with or without albumin, or by means of a disulphide affinity adsorbent (Sweet & Szabados, 1977).

The first application of affinity chromatography to the purification of a steroid-transforming enzyme was claimed by Nicolas *et al.* (1972); since then various soluble and particulate steroid dehydrogenases have been purified by means of a range of steroidal ligands, e.g. by Chin & Warren (1973), Benson *et al.* (1974), Sweet & Adair (1975), Aukrust *et al.* (1976), Hubert *et al.* (1976), Golf & Graef (1976) and Macdonald *et al.* (1976). Golf & Graef (1976) also used *p*-chloromercuribenzoic acid-Sepharose, whereas Heyns & De Moor (1974) purified a 3(17) β -hydroxy steroid

dehydrogenase from rat erythrocytes by affinity chromatography on Cibachron Blue F3G-A coupled to Sepharose.

Because all of the published procedures for the purification of steroid-transforming enzymes use substrate, cofactor or their analogues, the potential of a non-steroidal ligand was explored. Selection of such a compound was facilitated by the report of Langer *et al.* (1959) that the human placental oestradiol-17 β dehydrogenase (EC 1.1.1.62) has an absolute steric specificity for the 17 β -hydroxy group, that the steroid substrate must possess a highly planar ring A or B or both for significant reactivity, and that the enzyme interacted with the entire steroid surface. In view of these structural requirements, non-steroidal ligands with two benzene rings seemed worthy of investigation. A model for such a ligand is *p*-(*p*'-aminophenoxypropoxy)benzamidinium (Baker & Erickson, 1968). By linking this compound to CNBr-activated Sepharose, Jameson & Elmore (1971, 1974) obtained an affinity adsorbent for the column separation of bovine α - and β -trypsin. Subsequently, Hartree and his colleagues (Hartree, 1977) used the same adsorbent to purify ram acrosin and to separate stable from unstable forms of this enzyme (Brown & Hartree, 1978).

The strong positive charge of the amidinium group in this compound would appear to be superfluous, and possibly disadvantageous, in a non-steroidal ligand

for steroid dehydrogenases. We have therefore investigated the de-amidino form, namely *p*-(phenoxypropoxy)aniline (Brown & Hartree, 1978), using three steroid-transforming enzymes and two that do not react with steroid substrates.

Materials and Methods

Chemicals

Nicotinamide nucleotides and bovine serum albumin (crystallized and freeze-dried) were bought from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Steroid substrates were obtained commercially and purity was ascertained by melting point and t.l.c. in the systems described below. Steroids were recrystallized where necessary from ethanol or methanol. Reference compounds were obtained from the

M.R.C. Steroid Reference Collection (U.K.) by courtesy of Professor W. Klyne or Professor D. N. Kirk. 2-Acetamido-2-deoxy- β -D-glucopyranoside was bought from Koch-Light (Colnbrook, Bucks., U.K.). Sepharose 4B, CNBr-activated Sepharose and 2',5'-ADP-Sepharose 4B were produced by Pharmacia Fine Chemicals, Uppsala, Sweden. *p*-(Phenoxypropoxy)aniline hydrochloride was kindly provided by Dr. E. F. Hartree, A.R.C. Institute of Animal Physiology, 307 Huntingdon Road, Cambridge, U.K.

Enzymes

Oestradiol-17 β dehydrogenase (EC 1.1.1.62) was prepared by $(\text{NH}_4)_2\text{SO}_4$ precipitation from chicken liver as described by Renwick & Engel (1967) and was further purified by chromatography at room

Fig. 1. Chromatographic behaviour of oestradiol-17 β dehydrogenase and β -N-acetylglucosaminidase on *p*-(phenoxypropoxy)-aniline-Sepharose

Oestradiol-17 β dehydrogenase from chicken liver was prepared as described by Renwick & Engel (1967). The 0–30% satd. $(\text{NH}_4)_2\text{SO}_4$ precipitate was further purified by chromatography on hydroxyapatite as described in the text; this preparation also contained β -N-acetylglucosaminidase activity. After dialysis against starting buffer, the sample, which contained 2.6 units of oestradiol-17 β dehydrogenase activity and 40 mg of protein, was applied to a column (0.9 cm $\times$ 13 cm) of *p*-(phenoxypropoxy)aniline-Sepharose, which was then washed in the same buffer. Oestradiol-17 β dehydrogenase (●) and β -N-acetylglucosaminidase (▲) were assayed as described in the Materials and Methods section; the dehydrogenase was eluted in a linear gradient (starting at arrow) of KCl (----). The elution of protein was monitored at 280 nm (○) and 2 ml fractions were collected.

temperature (18–20°C) on a column (15 cm × 2.5 cm) of hydroxyapatite (Anacker & Stoy, 1958) in 5 mM- $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer, pH 6.8, containing 1 mM-mercaptoethanol. The enzyme was eluted in a linear gradient of phosphate (5–200 mM), containing 1 mM-mercaptoethanol; active fractions were collected in a Büchler refrigerated fraction collector, pooled and stored at –15°C after the addition of glycerol (50%, v/v). This preparation also contained small amounts of oestradiol-17 α dehydrogenase (EC 1.1.1.148), epitestosterone dehydrogenase and testosterone dehydrogenase (EC 1.1.1.64) as well as the lysosomal acid hydrolase, β -N-acetylglucosaminidase (EC 3.2.1.30).

3(17) β -Hydroxy steroid dehydrogenase (EC 1.1.1.-51) (grade II) from *Pseudomonas testosteroni* and cortisone reductase (EC 1.1.1.53) (type II) from *Streptomyces dehydrogenans* were bought from Sigma. Lactate dehydrogenase (EC 1.1.1.27) from rabbit muscle was from Calbiochem, San Diego, CA, U.S.A.

Enzyme assays

Steroid dehydrogenases were assayed under optimal conditions in a Unicam SP. 1800 spectrophotometer with constant-temperature cell holder. The avian enzymes were determined in a total reaction mixture of 3 ml by the method of Renwick & Engel (1967), modified to include 5 mg of bovine serum albumin. The assay temperature was 37°C.

The 3(17) β -hydroxy steroid dehydrogenase and cortisone reductase respectively were measured at 25°C in the following systems: 2 ml of water; 0.6 ml of 0.1 M-sodium pyrophosphate, adjusted to pH 8.9 with HCl; 0.1 ml of enzyme preparation; 1.2 μ mol of NAD^+ in 0.2 ml of water, adjusted to pH 7 with NaHCO_3 and 0.1 ml of ethanol containing 0.052 μ mol of testosterone; 2.1 ml of water; 0.6 ml of 0.2 M- $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer, pH 7.6, 0.1 ml of enzyme preparation, 0.6 μ mol of NADH in 0.1 ml of 0.2 M-phosphate buffer, pH 7.6, and 0.2 μ mol of cortisone in 0.1 ml of ethanol. All reaction mixtures were pre-incubated at the appropriate temperature for 3 min before the addition of substrate. Rates of increase or decrease in A_{340} were measured against suitable reference cells and initial velocities were determined.

β -N-Acetylglucosaminidase was measured as described by Willcox & Renwick (1977) in an Aminco-Bowman spectrophotofluorimeter set at 358 nm excitation and 448 nm emission. Lactate dehydrogenase was determined as described by Bergmeyer *et al.* (1965).

Enzyme units

Enzyme units are defined as the amount of enzyme that catalyses the transformation of 1 μ mol of substrate/min.

Thin-layer chromatography

Steroid substrates were regularly checked for purity by t.l.c. in the systems diethyl ether/benzene (2:1, v/v) and ethyl acetate/benzene (2:1, v/v).

Chromatograms were examined in the u.v. at 254 nm before spraying with ethanol/ H_2SO_4 (sp.gr. 1.84) (1:1, v/v). Steroids were recognized as coloured spots after the plates had been heated at 100°C for 5–10 min.

Tentative identification of the products of enzyme-catalysed steroid transformations was also carried out from time to time by combining reaction mixtures, extracting steroids with organic solvents and then comparing their mobilities against those of reference compounds in appropriate t.l.c. systems. Further characterization was obtained by chromatography of acetylated derivatives on 3% OV-1/2Q at 260°C in a Pye Series 104 gas-liquid chromatograph fitted with dual flame-ionization detectors.

Preparation of ligand and chromatographic columns

The method was that of Jameson & Elmore (1974) modified by E. F. Hartree (personal communication) as follows: 50 ml of a suspension of CNBr-activated Sepharose 4B was washed once with 2 M- Na_2CO_3 and

Table 1. Recovery of oestradiol-17 β dehydrogenase after 'affinity' chromatography on *p*-(phenoxypropoxy)aniline-Sepharose

Oestradiol-17 β dehydrogenase from chicken liver was prepared by $(\text{NH}_4)_2\text{SO}_4$ precipitation and further purified by chromatography on hydroxyapatite as described in the text before application to columns of *p*-(phenoxypropoxy)aniline-Sepharose, by the method described in the Materials and Methods section. The enzyme was assayed by the rate of formation of NADPH measured at 340 nm. Chromatography was carried out at ambient temperatures and salt gradients were formed by an LKB Ampholine gradient mixer. Protein concentrations were determined by the method of Lowry *et al.* (1951). The mean recovery was $53 \pm 14\%$ ($\pm$ S.E.M. for eight experiments); the mean overall purification was 13 ± 7 -fold ($\pm$ S.E.M. for eight experiments).

Enzyme applied (munits)	Adsorbed enzyme recovered (munits)	Recovery (%)	Overall purification (-fold)
2610	1135	43	24
2060	857	42	25
647	277	43	10
1428	616	43	11
916	614	67	10
840	633	75	13
1392	605	43	3
835	564	67	9

resuspended in this solution to give a volume of 100 ml. The gel was washed with 800 ml of cold 0.1 M-NaHCO₃ and resuspended in 0.1 M-sodium acetate, pH 5.0. The pH was adjusted to 6 by the dropwise addition of acetic acid, and 14 mg of *p*-(phenoxypropoxy)aniline hydrochloride was added in 5 ml of methanol/water (1:1, v/v). The suspension was stirred overnight at room temperature, then filtered and washed with 0.1 M-sodium acetate. Any remaining active groups were made to react with 1 M-ethanolamine, pH 8.0, for 2 h at room temperature. (Subsequent experiments showed that this step could be omitted.) The suspension was transferred to a glass column and washed slowly (approx. 80 ml/h) with 1 litre of 1 M-sodium acetate (pH 5.0) followed by 1 litre of 0.2 M-NH₄Cl/NH₃, pH 9.5, and water. The column was equilibrated with starting buffer (10 mM-Tris/HCl, pH 7.4, containing 1 mM-EDTA and 1 mM-mercaptoethanol). Evidence of coupling was obtained by measuring the A_{263} of *p*-(phenoxypropoxy)aniline in solution before and after reaction with CNBr-

activated Sepharose. About 90% of the ligand was attached to the Sepharose, although this value fell to about 73% when the column was washed preparatory to chromatography.

Columns of Sepharose 4B and CNBr-activated Sepharose 4B were used in control experiments. All columns were packed and run at room temperature, unless otherwise stated, by using non-jacketed glass columns. Gradients were formed by an LKB Ampholine gradient mixer with Ampholine stirrer motor. Column eluates were monitored at 280 nm and fractions were collected in a Büchler refrigerated fraction collector or in an LKB RediRac 2112. Conductivity was measured with a Radiometer conductivity meter (type CDM2e).

Protein content was determined by the method of Lowry *et al.* (1951) against reference solutions of bovine serum albumin.

Enzyme preparations were concentrated when necessary by means of a Sartorius Membranfilter collodion bag.

Fig. 2. Chromatographic behaviour of oestradiol-17 β dehydrogenase and β -N-acetylglucosaminidase on 2',5'-ADP-Sepharose. A fraction of chicken liver prepared as described in the Materials and Methods section was applied to a column (0.9 cm $\times$ 13 cm) of 2',5'-ADP-Sepharose. The sample contained 1.3 units of oestradiol dehydrogenase and 29 mg of protein. Oestradiol dehydrogenase (●) and β -N-acetylglucosaminidase (▲) were assayed as described in the text and the dehydrogenase was eluted in a 0–1 mM gradient (starting at arrow) of NADP⁺ (100 ml). Fractions (3.4 ml) were collected and protein (○) was measured at 280 nm.

Results and Discussion

The chromatographic behaviour of avian oestradiol-17 β dehydrogenase and β -*N*-acetylglucosaminidase from the same tissue is shown in Fig. 1, where a column of *p*-(phenoxypropoxy)aniline-Sepharose 4B was deliberately overloaded. A sharp separation of oestradiol dehydrogenase was obtained from the acid hydrolase and from the bulk of the protein applied. The oestradiol dehydrogenase activity associated with the front is thought to have resulted from the overloading, as no evidence for the existence of more than one species of enzyme was found in subsequent experiments. Retention of the oestradiol dehydrogenase contrasted sharply with its behaviour on 'blank' columns of Sepharose 4B and CNBr-activated Sepharose 4B, where this enzyme was not retarded.

The oestradiol-17 β dehydrogenase was also retained by *p*-(phenoxypropoxy)aniline-Sepharose when glycerol (10%, v/v) replaced 2-mercaptoethanol in the buffer used for equilibration and elution. When chromatography was performed at 4°C, a slightly greater separation of enzyme activity from the protein peak was obtained which was probably due to slower elution in the cold. The application of a steep salt gradient (10–200mM) or an abrupt increase in salt concentration was necessary to elute adsorbed pro-

tein, to ensure sharp peaks of narrow band width and to obviate 'trailing'. Recoveries of oestradiol-17 β dehydrogenase are listed in Table 1 and compare favourably with results published from other laboratories when steroid-transforming enzymes have been purified with steroidal ligands (e.g. Nicolas *et al.*, 1972; Benson *et al.*, 1974). Overall purifications ranged from 3- to 25-fold.

Oestradiol-17 β dehydrogenase preparations from several 'affinity' columns were combined, concentrated and assayed for oestradiol-17 α , epitestosterone and testosterone dehydrogenase activities; all were present. Similar results were obtained by affinity chromatography of oestradiol-17 β dehydrogenase on 2',5'-ADP-Sepharose 4B (Fig. 2), when the enzyme was eluted at a concentration of 0.15mM-NADP⁺. However, the bacterial 3(17) β -hydroxy steroid dehydrogenase was not bound to the ligand under the same conditions, whereas cortisone reductase from *Streptomyces dehydrogenans* was retained (Fig. 3).

Because Langer *et al.* (1959) found an apparent correlation between the degree of planarity of the steroid substrate and its affinity for and reactivity with the oestradiol-17 β dehydrogenase of human placenta, we had postulated that the availability of an aromatic ring on a hydrophobic leash in *p*-(phenoxypropoxy)aniline might mimic ring A of the

Fig. 3. Chromatographic behaviour of cortisone reductase on *p*-(phenoxypropoxy)aniline-Sepharose. Cortisone reductase from *Streptomyces dehydrogenans* (1.1 units) was applied to a column (1.6cm $\times$ 14cm) of *p*-(phenoxypropoxy)aniline-Sepharose, which was washed with starting buffer. Enzyme activity (Δ) was eluted in a linear gradient (starting at arrow) of KCl (----), and assayed as described in the text. Fractions (3.9ml) were collected and protein ($\circ$) was measured at 280nm.

steroid substrate for the avian oestradiol dehydrogenase. The results do not support this hypothesis; the 3(17) β -hydroxy steroid dehydrogenase was not adsorbed, yet this enzyme is known to oxidize oestradiol-17 β or testosterone as well as a number of other steroid secondary alcohols (Talalay, 1962). Moreover, preparations of oestradiol-17 β dehydrogenase from several 'affinity' columns all contained oestradiol-17 α dehydrogenase, epitestosterone dehydrogenase and testosterone dehydrogenase activi-

ties and this observation does not support exclusive, sterically specific linkages between ligand and steroid-binding site(s) on the enzyme.

It was also found that cortisone reductase was preferentially adsorbed from a mixture (Fig. 4) which suggests that steroid-transforming enzymes are most likely to be adsorbed if they react with polar substrates. Apart from the increasing polarity of testosterone, oestradiol-17 β and cortisone, inspection of molecular models of these steroids does not reveal

Fig. 4. Chromatographic behaviour of a mixture of steroid dehydrogenases on *p*-(phenoxypropoxy)aniline-Sephadex. A mixture of oestradiol-17 β dehydrogenase (●) prepared as described in the legend to Fig. 1, bacterial 3(17) β -hydroxysteroid dehydrogenase (■) and cortisone reductase (Δ), 0.89, 1.1 and 0.99 units respectively, was applied to a column (0.6 cm $\times$ 14 cm) of *p*-(phenoxypropoxy)aniline-Sephadex. Elution was carried out with starting buffer before a linear gradient (starting at arrow) of KCl (----) was applied. Enzyme activities were determined as described in the text. Fractions (4 ml) were collected and protein (○) was measured at 280 nm.

any common features which might account for the preferential adsorption of cortisone reductase or oestradiol dehydrogenase; however, one must be cautious when attempting to adduce evidence from experiments with enzymes from different species.

It is most unlikely that the ligand acts by virtue of structural similarities to the nicotinamide nucleotides used as cofactors by the steroid dehydrogenases in our experiments; the ligand is hydrophobic, the nucleotides hydrophilic. This premise was upheld experimentally when it was found that the chromatographic behaviour of the steroid dehydrogenases bore no relation to cofactor requirement. Cortisone reductase, which was preferentially adsorbed from a mixture (Fig. 4), uses NADH as cofactor, whereas the avian oestradiol-17 β dehydrogenase is NADP-linked and the bacterial 3(17) β -hydroxy steroid dehydrogenase uses either nicotinamide nucleotide. We were therefore surprised to find that the NAD-dependent lactate dehydrogenase was adsorbed on columns of *p*-(phenoxypropoxy)aniline, and eluted with KCl, in

a manner similar to that found on chromatography of the same enzyme on 2',5'-ADP-Sephacrose. When the oestradiol dehydrogenase was chromatographed on columns of *p*-(phenoxypropoxy)aniline-Sephacrose, the enzyme could be eluted in NADP gradients; recoveries were less than those obtained with salt gradients.

The most plausible explanation for the chromatographic behaviour of the various dehydrogenases on this non-steroidal ligand is that bonds formed between enzyme and *p*-(phenoxypropoxy)aniline are not of a single type (i.e. there are not exclusively stereospecific linkages between ligand and steroid-binding site on the enzyme). A predominantly hydrophobic ligand, as in this case, can bind proteins by hydrophobic interactions with apolar regions of the protein surface; such binding is non-specific and bond strengths are heterogeneous. Hofstee (1973) has shown that hydrophobic bonding to an apolar ligand is affected by electrostatic or other polar forces possibly involving the supporting matrix.

Fig. 5. Chromatographic behaviour of 3(17) β -hydroxy steroid dehydrogenase on *p*-(phenoxypropoxy)aniline-Sephacrose in 3 M-KCl

3(17) β -Hydroxy steroid dehydrogenase from *Pseudomonas testosteroni* (2.75 units) was applied to a column (1.6 cm $\times$ 30 cm) of *p*-(phenoxypropoxy)aniline-Sephacrose in starting buffer containing 3 M-KCl. The column was washed with this mixture before enzyme activity (■) was eluted in a decreasing gradient (starting at arrow) of KCl (---) and assayed as described in the text. Fractions (4 ml) were collected and protein (○) was determined at 280 nm.

In our experiments, release of adsorbed enzyme from the hydrophobic ligand by a salt gradient of increasing concentration was necessary, which, according to Hofstee (1973), is evidence that electrostatic interaction with the possible positive charge on the matrix-bound amino nitrogen of the substituting ligand plays a major role in the binding. When *p*-(phenoxypropoxy)aniline is coupled to CNBr-activated Sepharose, a secondary amine group is formed which will be positively charged at the pH values used in these experiments. However, high ionic strengths not only suppress electrostatic binding, they also enhance hydrophobic bonding (Hofstee, 1975), because some proteins will bind to a hydrophobic ligand only at high ionic strength (e.g. in 3M-NaCl), and for this to occur, hydrophobic interaction must be predominant and electrostatic interaction relatively unimportant. Hofstee's (1975) data suggested little or no specificity of binding. For the bacterial 3(17) β -hydroxy steroid dehydrogenase the enzyme was not adsorbed by the ligand except when the salt concentration was increased to 3M (Fig. 5). The effect was reversed on decreasing the concentration of KCl. In addition to the factors mentioned it is possible that aromatic rings are important in binding. Nishikawa & Bailon (1975) not only found that α -chymotrypsin and β -galactosidase were bound to hydrophobic ligands, but observed stronger binding when the ligands contained aromatic rings.

When *p*-(phenoxypropoxy)aniline is used we might expect the formation of specific bonds between ligand and active sites of enzymes, non-specific apolar bonding (which may be unrelated to enzyme activity or active sites), and ionic bonding which involves the secondary amine group.

p-(Phenoxypropoxy)aniline clearly offers promise for the purification of steroid-transforming enzymes where elution with steroid substrate or cofactor is not wanted. Use of this ligand obviates the need for elution with expensive nucleotides and dispenses with the use of steroid substrates, which may interfere with subsequent assays, besides posing problems of solubility in aqueous solutions. Moreover, removal of the enzyme from the column is readily accomplished without resort to hydrolysis (Chin & Warren, 1973). *p*-(Phenoxypropoxy)aniline may also be of use in the purification of lactate dehydrogenase and other enzymes with or without manipulation of the ionic strength of buffers used in chromatography, and the ligand may be of service in the purification of receptors for hormonal steroids under mild conditions at ambient temperatures or at 4°C. Problems associated with the purification of such receptors by affinity chromatography have been emphasized by Cuatrecasas (1972), Ludens *et al.* (1972) and Sica *et al.* (1973).

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